Remarks/Arguments

By the present amendment, claim 1 and claims dependent thereon have been amended to specify that OP9 cells have been modified to express a Notch ligand. Claim 1 and claims dependent thereon have been further modified to exclude TCR-$\alpha\beta^+$ $CD4^+CD8^-$ T cells from the claimed subject matter. Claim 8 has been amended to correct a typographical error. Claims 29-43 have been deleted and new claims 44-49 have been introduced. New claims 44-46 claim a system that produces specific subsets of T cells and methods of forming and expanding said subsets of T cells. Support for these claims is provided at pg. 22 lines 1-10 and by Examples 2, 3,5, 7, 9 and10. New claims 47-49 claim the formation of CD4+CD8- T cells where the stem cells or progenitor cells are cultured with a system of claim 1, isolated and transferred to a recipient animal with a thymus. Support for these claims is provided by Examples 8 and 11 of the specification which show that CD4+CD8- T cells can be generated from *in vitro*-derived progenitor T cells if these cells are then transferred to an animal with a thymus (pg. 44, line 29; pg. 58 line 4).

The amendments to the claims have been made without prejudice and without acquiescing to any of the Examiners objections. Applicant reserves the right to pursue any of the deleted subject matter in a further divisional, continuation or continuation-in-part application. The amendment does not contain new matter and its entry is respectfully requested.

The Official Action dated July 28, 2005 has been carefully considered. It is believed that the amended specification and claims and the following comments represent a complete response to the Examiner's rejections and place the present application in condition for allowance. Reconsideration is respectfully requested.

I. 35 USC§112, First Paragraph

The Examiner has objected to claims 1,2,4,8,10-17,22,24 and 29-43 under 35 USC§112, first paragraph for lack of an enabling disclosure for the development of all mature T cell subsets, specifically TCR-$\alpha\beta^+$ $CD4^+CD8^-$ T cells. Claims 29-43 have been canceled hence the stated objection with regards to these claims is no longer applicable. Claims 1,2,4,8,10-17, 22 and 24 have been amended to exclude TCR-$\alpha\beta^+$ $CD4^+CD8^-$ T cells from the claimed subject matter.

New claims 44-46 have been added which claim a system that produces T cell subsets: (a) $CD4^-$ $CD8^-$ $CD25^+$ $CD44^{+/-}$ double negative (DN) T cells; (b) TCR-$\alpha\beta^+$ $CD4^+CD8^+$ double positive (DP) T cells; (c) TCR-$\alpha\beta^+$ $CD4^-CD8^+$ T cells; and (d) TCR-$\gamma\delta^+$ T cells; as well as methods of forming and expanding these subsets of T cells. Support for these claims is provided at pg. 22 lines 1-10 and by Examples 2, 3,5, 7, 9 and 10.

New claims 47-49 have been added which claim the formation of TCR-$\alpha\beta^+$ CD4+CD8- T cells wherein stem cells or progenitor cells are cultured with a system of claim 1, isolated and transferred to a recipient animal with a thymus. Support for these claims is provided by Examples 8 and 11 of the specification which show that TCR-$\alpha\beta^+$ CD4+CD8- T cells can be generated from *in vitro*-derived progenitor T cells if these cells are then transferred to an animal with a thymus (pg. 44, line 29; pg. 58 line 4).

In view of the foregoing we respectfully submit that the application enables one skilled in the art to generate the T cell lineages claimed in a manner commensurate with the breadth of the claims.

In view of the foregoing, we respectfully request that all of the objections to the claims under 35 USC§112, first paragraph, be withdrawn.

II. 35 USC §112, Second Paragraph

The Examiner has objected to claims 1,2,4,8,10-17,22,24 and 29-43 under 35 USC§112, second paragraph, as being incomplete for omitting essential elements, the omitted elements being the transfection of OP9 or the mouse stromal cells with a vector encoding DL-1 or DL-4. Claims 29-43 referring to mouse stromal cells have been canceled, consequently the objection to these claims no longer applies.

Regarding claims 1,2,4,8,10-17,22 and 24, the specification does not disclose that OP9 cells must be transfected *per se* but rather that such cells must be modified to express DL-1 or DL-4 in order to induce T cell lymphopoiesis but not induce B cell lymphopoiesis. DL-1 or DL-4 expression in OP9 cells can be achieved by a number of methods including but not limited to transfection, retroviral transduction, genetic engineering and microinjection. The specification discloses at pg. 49 lines 5-10 that Jaleco et al. transduced S17 cells with retroviruses capable of expressing Delta-1 and at pg. 29 lines 26-29 that the inventors used a retroviral vector engineered to express DL-1 or DL-4 to infect OP9 cells. Consequently, claims 1,2,4,8,10-17,22 and 24 have been amended to specify that the *in vitro* system comprises OP9 stromal cells that have been modified to express a Notch ligand wherein the Notch ligand is DL-1 or DL-4.

In view of the foregoing, we therefore respectfully submit that transfection *per se* of OP9 cells is not an essential element of the invention and that the claims as currently amended include all the essential elements of the invention.

In view of the foregoing, we respectfully request that all of the objections to the claims under 35 USC §112, second paragraph, be withdrawn.

III. 35 USC §103(a)

The Examiner has objected to claims 1,2,4,8, 12-15, 17, 22, 29-33, 36-39, 41-42 as being obvious in light of Jaleco et al. (Jaleco et al. (2001) *J. Exp. Med.* 194:991-1001), Nakano et al. (Nakano et al. (1994) *Science* 265:5175), and Tatsumi et al. (Tatsumi et al. (1990) *Proc. Natl. Acad. Sci.* 87:2750-2754). Claims 29-33, 36-39, and 41-42 have been canceled, consequently the above objection to these claims no longer applies. Regarding claims 1,2,4,8,12-15,17 and 22, we respectfully submit that the information disclosed by the aforementioned references fails to provide the necessary disclosure or motivation required for the generation and expansion of T cells from stem cells and progenitors using OP9 cells modified to express DL-1 or DL-4.

The present invention relates to a novel system for generating and expanding mature T cells from stem cells and progenitor cells. Prior to the present invention, generating T cells *in vitro* "required cumbersome fetal thymic organ culture systems or reaggregates of thymic epithelial cells and stem cells, that recapitulate the 3-dimensional structure of the thymus (Lehar SM and Bevan MJ, "T Cell Development in Culture" *Immunity* 17:689-92) a copy of which we enclose. Although *in vitro* systems to produce cells of erythroid, myeloid and B-cell lineages from stem cells existed (Nakano et al.), attempts to produce mature T cells *in vitro* from progenitor cells had failed (Jaleco et al.).

The present invention overcomes the problems of the prior art by providing a simple *in vitro* method that permits the generation of mature T cells from stem cells and progenitor cells. The novelty of the claimed invention is supported by a mini-review of the technology in the prestigious journal *Immunity* (Lehar SM and Bevan MJ, "T Cell Development in Culture" *Immunity* 17:689-92), a copy of which we enclose. The authors describe the system of the subject invention as "a novel *in vitro* culture system for T cell development" (Lehar and Bevan, pg. 691). They commented that the inventors of the subject invention provide "a previously unavailable, easily manipulable culture system that will undoubtedly facilitate future studies" (Lehar and Bevan, pg. 689). None of the prior art cited by the

Examiner, either alone or in combination, would lead one of ordinary skill in the art to the present invention.

As the Examiner is aware, he has the burden of factually supporting any *prima facie* conclusion of obviousness. To establish a *prima facie* case of obviousness, three basic criteria must be met. First, the prior art references when combined must teach or suggest all the claim limitations. Second, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Finally, there must be a reasonable expectation of success. We respectfully submit that the above criteria have not been met as explained below.

1) Prior art does not teach all of the claimed limitations

Jaleco et al., Nakano et al. and Tatsumi et al. do not teach all of the claimed limitations.

First, the cited references do not teach a system for the generation of mature T cells that are produced using stem cells or progenitor cells. Second, the cited references do not teach a system that uses modified OP9 cells to generate mature T cells. Third, the cited references do not teach a system that uses DL-1 or DL-4 to produce mature T cells. Finally, the cited references do not teach a system that provides a method for cell expansion.

The cited references do not teach a system for the generation of mature T cells that are produced using stem cells or progenitor cells. Jaleco et al. uses progenitor cells but is unable to produce any mature T cells. This lack of functionality was reiterated in the review of Lehar and Bevan where the authors stated that Jaleco et al. "were unable to generate any mature T cells" (Lehar and Bevan, p689). The system described by Nakano et al. does not permit the generation of any T cell lineage and therefore cannot teach a method for the

generation of mature T cells. Only Tatsumi et al. teaches the generation of mature T cells. However, the precursor cells used in Tatsumi et al. were not stem cells or uncommitted progenitor cells. The method claims of the present invention are limited to precursor cells that are stem cells or progenitor cells. Therefore none of the cited references were able to achieve the present invention wherein the generation of mature T cells is achieved using stem cells or progenitor cells.

Second, the cited references do not teach a system that uses modified OP9 cells to generate mature T cells. Jaleco et al. and Tatsumi et al. do not use modified or unmodified OP9 cells. Nakano et al. does not use modified OP9 cells; they use only unmodified OP9 cells.

Third, the cited references do not teach a system that uses DL-1 or DL-4 to generate mature T cells. Nakano et al. and Tatsumi et al. do not use DL-1 or DL-4. Jaleco et al. uses Delta-1. However, as mentioned above, Jaleco et al. were unable to generate any mature T cells.

Furthermore the cited references do not teach a system that provides for expansion of T cells. The present invention provides an *in vitro* system which produces increased numbers of T cells. In an example the present inventors saw an increase in cellularity of 100 fold in the first week of culture and another 15-20 fold expansion by day 12 (Specification, Example 2, pg. 33 lines 19-21 for support). Jaleco et al. observes that cellular expansion was considerably lower for cells cultured with Delta-1 stromal cells (Jaleco et al., pg.995). Tatsumi et al. used short-term cultures (1-3 days) and did not report increased T cell numbers whereas the system of Nakano et al. did not produce any T cells.

Therefore the cited references clearly do not teach the claimed limitations as they are not directed to the *in vitro* generation of mature T cells or to the *in vitro* expansion of T cells from stem cells and progenitor cells.

2) No suggestion or motivation in the prior art

Jaleco et al., Nakano et al. and Tatsumi et al. provide no motivation to one of ordinary skill in the art to modify their teachings in order to achieve the present invention.

The Examiner suggests that it would be *prima facie* obvious to modify the teachings of Jaleco et al. by replacing mouse S17 stromal cells with the OP9 cells of Nakano et al. We respectfully disagree. Nakano et al. does not teach a system that generates *any* T cells. Nakano et al. was directed to circumventing the almost exclusive production of macrophages from ES cells in their system. They found that OP9 cells, which do not produce functional M-CSF, permitted the development of myeloid, erythroid and B-cells whereas other stromal cells resulted in the almost exclusive development of macrophages. The system of Nakano et al. was not directed at generating *any* T cells from stem cells or progenitor cells and did not address whether or how the system could be modified to produce mature T cells. Consequently Nakano et al. provides no guidance to one of ordinary skill in the art on how to produce mature T cells from stem cells or progenitor cells.

Further, no motivation on how to achieve the present invention was provided by Jaleco et al. Jaleco et al. was directed to modifying B cell lineage development using stromal cells expressing Delta-1. Coculturing progenitor cells with Delta-1 expressing stromal cells gave rise to cells the authors described as "having features compatible with those of T/NK cell progenitors" (Jaleco et al., pg. 998). There is no guidance in Jaleco et al. on how to produce mature T cells from stem cells or progenitor cells. Additionally, there is no guidance in Jaleco et al. to seek out M-CSF deficient stromal cells such as OP9. The system of Jaleco et al. was not affected by the problem encountered by Nakano et al., namely the almost exclusive differentiation of cells into macrophages. Therefore there was no basis to consider the effect of M-CSF expression and consequently no motivation to use a stromal cell line deficient in M-CSF. Consequently, there is no reason why

someone of ordinary skill in the art would be motivated to replace the S17 stromal cells taught in Jaleco et al. with the OP9 cells used in Nakano.

Tatsumi et al. also provides no motivation to achieve the present invention. Tatsumi et al. is directed at inducing differentiation of immature thymocytes not stem cells or progenitor cells. The precursor cells used by Tatsumi et al. were a mixed population of CD4-CD8- thymocytes which are already committed to the T cell lineage. There is no guidance as to what modifications would be necessary to generate mature T cells from stem cells or progenitor cells. Furthermore Tatsumi et al. use a thymic stromal cell clone. The abstract concludes by stating "immature thymocytes are promoted to differentiate ... *by* a specialized thymic stromal component (Tatsumi et al., pg. 2750). The authors report that non-thymic bone marrow derived stromal cells were *unable* to induce T cell differentiation (Tatsumi et al., pg. 2754). The present invention uses non-thymic OP9 stromal cells derived from newborn calvaria (i.e. skull cap; Nakano et al, pg. 1098). Tatsumi et al. therefore teaches away from the current invention by concluding that thymic stromal cells are necessary for T cell development. Hence there is no direction and no motivation in Tatsumi et al. to modify the teachings of Jaleco et al. by using OP9 cells.

3) No reasonable expectation of success

None of the cited references would provide one of ordinary skill in the art a reasonable expectation of success in achieving the present invention.

Even if a person of ordinary skill in the art would be motivated to substitute OP9 cells of the Nakano et al. system, which were known to promote erythroid, myeloid and B cell lineage development, for the S17 cells of Jaleco et al., a person of ordinary skill in the art would not have a reasonable expectation of success.

The Nakano system required further modifications before it was able to support effective T cell differentiation using OP9 cells expressing DL-1. These modifications included the addition of specific cytokines to the culture system. Therefore further experimentation that was not routine or obvious to a person of ordinary skill in the art was required before OP9 cells expressing DL-1 were able to support the induction and differentiation of T cells from defined sources of stem cells. In light of the foregoing we submit that it would not be obvious at the time of the invention to combine the teachings of Jaleco et al. and Nakano et al.

Additionally, a person of ordinary skill in the art employing the system of Jaleco et al. using OP9 stromal cells would not have a reasonable expectation of success due to the reduced purity of progenitor cells and differences in culturing procedure employed. The inventors of the present invention used CD34+ cells that were >99% pure whereas Jaleco et al. used CD34+ cells that were only 80-95% pure (Jaleco et al., pg. 992) and unlike the present inventors, Jaleco et al. did not see considerable cell expansion using Delta-1 expressing S17 cells. Since only approximately 1 in 17 progenitor cells gives rise to T cells using the system of the present invention (Specification, pg. 48 line 16), the culturing procedure employed in Jaleco et al. would not lead one of ordinary skill in the art to have a reasonable expectation of achieving the present invention.

In summary, we respectfully submit that the Examiner has not met the burden of establishing obviousness in the present case as none of the above criteria have been satisfied.

We remind the Examiner that he is to consider secondary considerations when assessing obviousness. Secondary considerations relevant to the present invention include long-felt need in the art. The in vitro generation of mature T cells is highly desirable. However, as stated previously, there are difficulties recognized the art with respect to the simple in vitro production and recovery of T cell subsets. The present invention solves the difficulties of the prior art by

providing an efficient system for generating mature T cells. Therefore, there is a clear long felt need in the art for the present invention which must be given due weight when considering inventive step.

In view of the foregoing, we respectfully request that all of the objections to the claims under 35 USC §103(a) be withdrawn.

The Commissioner is hereby authorized to charge any fee (including any claim fee) which may be required to our Deposit Account No. 02-2095.

In view of the foregoing comments and amendments, we respectfully submit that the application is in order for allowance and early indication of that effect is respectfully requested. Should the Examiner deem it beneficial to discuss the application in greater detail, he is kindly requested to contact the undersigned by telephone at (416) 957-1682 at his convenience.

Respectfully submitted,

BERESKIN & PARR

By M Gravelle

Micheline Gravelle
Reg. No. 40,261
Bereskin & Parr
Box 401, 40 King Street W
Toronto, Ontario
Canada M5H 3Y2
Tel: 416-957-1682
Fax: 416-361-1398

Attachment

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T Cell Development in Culture

Minireview

Sophie M. Lehar and Michael J. Bevan*
Department of Immunology and
Howard Hughes Medical Institute
University of Washington
Seattle, Washington 98195

The T cell compartment is continuously replenished by a renewable source of stem cells. In the adult, bone marrow-derived stem cells seed the thymus and initiate a developmental program that requires a series of incompletely understood signals that are normally provided by the thymus. Failure to recapitulate this process in simple in vitro cultures has hampered efforts to fully characterize these unique signals. In this issue of *Immunity*, Schmitt and Zúñiga-Pflücker describe a simple in vitro culture system that is able to generate mature T cells from fetal liver stem cells by expressing the Notch ligand Delta-1 on the OP9 stromal cell line. This finding should greatly enhance efforts to study T cell development and may provide a tool for generating defined T cell populations in vitro.

Lymphocytes develop from the descendents of multipotent hematopoietic stem cells (HSC) that have lost the ability to generate erythroid or myeloid progeny. One step along the way is referred to as the common lymphoid precursor (CLP), which can give rise to both B and T lymphocytes. In order to fully mature, both B and T lymphocyte progenitors must productively rearrange their antigen receptor genes through a highly ordered process that regulates their progression through distinct developmental stages (Rothenberg, 2000). In adult mice, B cell development occurs predominantly in the bone marrow (BM), while T cell development is restricted almost exclusively to the thymus. Although the various stages of B cell development have been successfully reproduced in vitro (Rolink et al., 1995), to date, the generation of T cells in vitro has required cumbersome fetal thymic organ culture systems, or reaggregates of thymic epithelial cells and stem cells, that recapitulate the 3-dimensional structure of the thymus. This has created the notion that thymus architecture and stromal cell types provide unique signals that are essential for normal thymopoiesis (Hare et al., 1999).

Developing T cells are thought to migrate to distinct regions within the thymus as they mature (Figure 1). In a recent study, Lind et al. (2001) proposed that uncommitted CLPs enter the thymus at the cortico-medullary junction, migrate outward through the cortex as they initiate TCRβ rearrangement, and proliferate within the subcapsular region following β selection. $CD4^+CD8^+$ (DP) thymocytes are thought to undergo positive selection on cortical epithelial cells, while mature $CD4^+$ or $CD8^+$ (SP) thymocytes undergo negative selection as they traverse into the medulla. This dynamic movement of developing thymocytes suggests that distinct differentiative signals reside in microenvironments within the thymus (Anderson and Jenkinson, 2001), but it remains incompletely understood what these spatially restricted signals may be. Recent data suggest that at least some of these signals are delivered through the Notch signaling pathway.

In a report appearing in this issue of *Immunity*, Schmitt and Zúñiga-Pflücker demonstrate that by providing a Notch signal in a culture system that otherwise promotes B cell development, the entire program for T cell maturation to the mature T cell stage can be achieved in vitro. These data complement a recent report by Jaleco et al. (2001), which examined the role of different Notch ligands (Delta-1 or Jagged-1) in regulating the differentiation of human hematopoietic progenitors. Jaleco et al. presented intriguing data suggesting that different Notch ligands may exert unique effects on T cell lineage commitment, but they were unable to generate any mature T cells. Together, these reports strengthen the notion that Notch signaling regulates key checkpoints during T cell development, and may provide unique spatially restricted signals that are normally provided by the intact thymus. Although the data presented by Schmitt and Zúñiga-Pflücker are by no means the last word on T cell development, they describe a previously unavailable, easily manipulable culture system that will undoubtedly facilitate future studies aimed at characterizing the signals required throughout this complex pathway of differentiation.

Summary of the Notch Pathway

The Notch pathway is an evolutionarily conserved signaling mechanism that regulates developmental decisions in diverse organisms including worms, flies, and mammals by regulating cell-cell interactions between receptor and ligand-bearing cells. Notch signaling is thought to influence patterning during the development of complex organ systems, and can be activated sequentially during progressive developmental stages (Artavanis-Tsakonas et al., 1999). Four mammalian Notch receptors have been identified, which bind to a family of at least five different ligands that belong to either the Delta or Jagged/Serrate family (Figure 2). Notch receptors are large type I transmembrane proteins that share a unique signaling mechanism. Ligand binding induces a proteolytic cleavage within the Notch intracellular domain, to generate an active form of the receptor (Notch-IC) that translocates into the nucleus and activates transcription by interacting with a ubiquitously expressed transcriptional repressor CBF1/RBP-Jκ (CBF1). The highly conserved RAM and Ankyrin domains of Notch-IC displace transcriptional corepressors by binding directly to CBF1, while a distinct transcriptional activation domain (TAD) domain recruits transcriptional coactivators (Mumm and Kopan, 2000).

Notch Signaling Regulates T Cell Development

There is considerable evidence that signaling through Notch receptors regulates multiple stages of T cell development. A clear consensus is emerging that Notch1 regulates the earliest stages of T cell commitment by

*Correspondence: mbevan@u.washington.edu

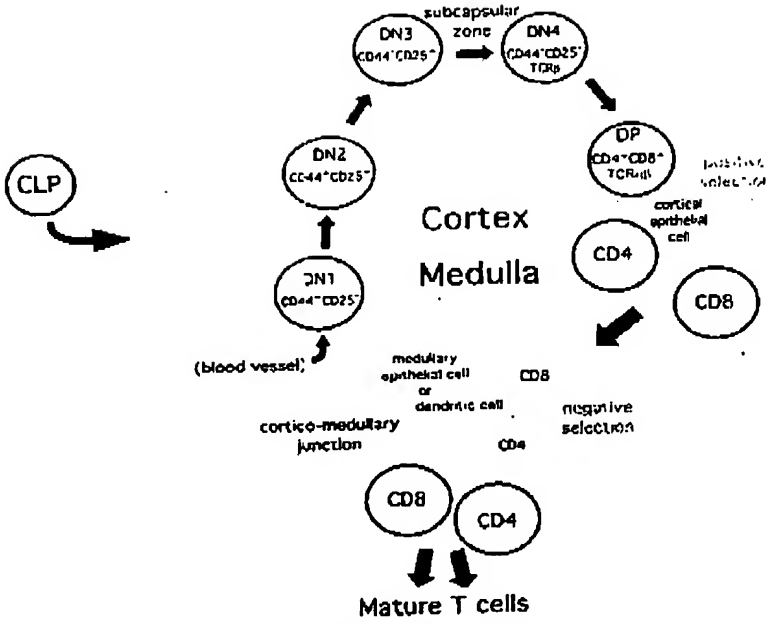

Figure 1. Migration of Maturing T Cells in the Thymus

providing a directive signal that promotes differentiation of CLPs into the T cell lineage, while inhibiting B cell differentiation (Izon et al., 2002; Radtke et al., 2002). Loss of Notch1 function in HSCs blocks T cell differentiation and results in the accumulation of immature B cells in the thymus. Conversely, gain of Notch1 function by overexpression of Notch-IC in HSCs blocks B cell development and results in T lineage commitment in the bone marrow. The Notch pathway has also been proposed to play a role in maintaining HSC populations, regulating thymocyte differentiation into the γδ *vs.* αβ lineages, or in promoting or inhibiting the maturation of immature $CD4^+$ or $CD8^+$ thymocytes.

Efforts to generate a consensus on how Notch signaling may influence these various functions has been aggravated by the inherent redundancy of the system. The four mammalian Notch receptors and their ligands are expressed in lymphoid organs in complex overlapping patterns that do not correlate directly with their proposed function. For example, although signaling through Notch1 is thought to influence T lineage commitment in the thymus, there is evidence that Notch1 is expressed on HSCs, thymocytes, and on maturing B cells (Anderson and Jenkinson, 2001; Bertrand et al., 2000). This apparent contradiction may be explained by the observation that Notch signals are subject to regulation by a number of molecules that can affect ligand binding or modulate intracellular signals. The ability of Notch receptors to respond to ligands of either the Delta or Jagged/Serrate class is influenced by a family of three different Fringe molecules (Lunatic, Manlac, and Radical Fringe). Interestingly, overexpression of Lunatic Fringe in thymocytes appears to inhibit Notch signals and results in increased B cell differentiation in the thymus

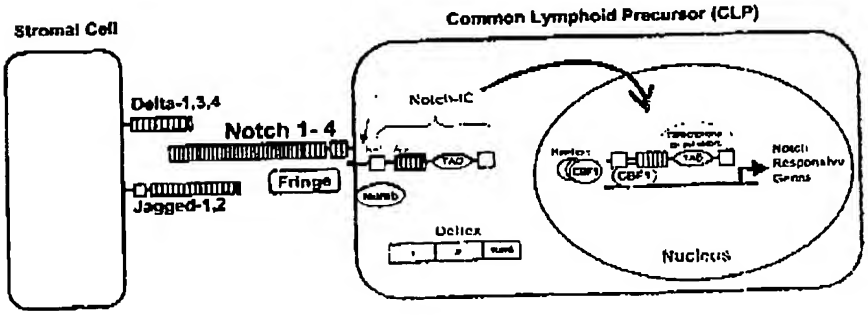

Figure 2. Receptors, Ligands, and Regulatory Molecules in the Notch Signaling Pathway

(Koch et al., 2001). At the intracellular level, Notch signals can be regulated by a number of cytoplasmic or nuclear proteins including Numb, Hairless, and Deltex. These molecules may regulate the intensity, duration, or quality of Notch signals (Radtke et al., 2002).

Finally, there is some evidence that Notch signals can be modified by the activity of other signaling pathways. For example, it has been proposed that signals through Notch1 and Notch2 are differentially affected by stimulation with M-CSF or GM-CSF (Milner and Bigas, 1999). Together these observations suggest that Notch signals may be interpreted differently by cells during specific developmental stages or under the influence of different micro-environmental conditions. With all of these factors coming to play, perhaps simultaneously on a single cell, there is a clear need for a simple in vitro system that will allow the contribution of individual components of the Notch signaling pathway to be examined separately.

A Novel In Vitro Culture System for T Cell Development

Schmitt and Zúñiga-Pflücker propose that the requirements for differentiation of T cells and B cells are remarkably similar. By expressing the Notch ligand Delta-1 on a stromal cell line, OP9-DL1, they were able to induce murine fetal liver stem cells (sorted $CD117^+/Sca1^{lo}/Lin^-$) to differentiate into what appear to be functionally mature $CD8^+$ T cells, under the influence of IL-7 and Flt3-L. Similar cultures containing unmodified OP9 stromal cells generated NK cells early in the culture period, and then exclusively B cells.

In the OP9-DL1 cultures, stem cells were induced to differentiate following a program that resembles that seen in the thymus. Within 12 days, cell numbers expanded more than 2,000-fold, and produced substantial numbers of both γδ and αβ lineage T cells. αβ lineage T cells appeared to initiate a complete developmental program including progression through the normal stages of CD4 and CD8 expression. By day 7, the normal $CD4^-CD8^-$ (DN) subsets were evident, as determined by expression of CD44, CD25, and TCRβ rearrangement. The authors reported (but did not show) that cultures seeded with $RAG^{-/-}$ stem cells underwent the expected developmental arrest at the DN3 stage. The above culture system should greatly facilitate future studies examining the specific requirements for cytokines or cell surface receptors during developmental progression between the DN stages. This analysis is particularly interesting in light of recent data from Wolfer et al. (2002), that suggested signaling through Notch1 is essential for complete TCRβ rearrangement and normal thymocyte progression through at least the DN2/DN3 stage.

Schmitt and Zúñiga-Pflücker also presented data suggesting that normal, functionally mature T cells could be generated in their OP9-DL1 cultures. The authors were able to generate a small number of $CD8^+$ T cells that expressed mature levels of surface TCR. At least some of these TCR^{hi} $CD8^+$ T cells appeared to be functionally mature in that they could be induced to synthesize IFNγ, following in vitro stimulation for 3 days with plate-bound anti-CD3. It was not clear however how this activation compared with that of normal $CD8^+$ T cells, as a control was not shown. Additionally, the authors did not attempt to generate mature $CD4^+$ T cells by providing MHC-II in their cultures.

It is not clear whether expression of Delta-1 on OP9 stromal cells is sufficient to induce the entire normal T cell developmental program, including positive and negative selection of functionally mature $CD4^+$ and $CD8^+$ T cells. Nevertheless, the above data clearly demonstrated that a normal T cell-specific developmental program was initiated and proceeded efficiently up to at least the $CD4^+CD8^+$ DP stage. Schmitt and Zúñiga-Pflücker estimated the progenitor frequency for T cells at approximately 1 in 17 stem cells in OP9-DL1 cocultures and at 1 in 6 for B cells in OP9 cocultures. The high frequency of T cell progenitors suggests that the observed T cell population resulted from differentiation of an uncommitted stem cell pool rather than the selective outgrowth of rare contaminating immature T cells.

Defining the Signals that Regulate T Cell Maturation

The above data presented by Schmitt and Zúñiga-Pflücker is similar to a previous study reported by Jaleco et al. which suggested that Delta-1, but not Jagged-1, can inhibit B cell commitment. Jaleco et al. employed a similar culture system utilizing a different stromal cell line (S17) and $CD34^+$ stem cells isolated from human cord blood. Both studies reported inhibition of B cell development in cultures containing stromal cells transfected with Delta-1. Jaleco et al. were able to generate significant numbers (up to 66%) of what appeared to be immature T/NK progenitors, expressing CD7 and intracellular CD3, and small numbers (up to 4%) of $CD4^+CD8^+$ T cells.

The striking finding in Jaleco et al. was the observation that two different Notch ligands, Jagged-1 and Delta-1, which are both expressed on thymic epithelial cells, are not equivalent. Only Delta-1 was able to inhibit B lineage commitment and promote differentiation of immature T cell precursors. But comparison of these two studies raises other important questions. For example, it will be important to determine why Jaleco et al. were unable to generate significant numbers of DP T cells. Inefficient T cell maturation could result from differences between human and murine stem cell populations, added growth factors, or unique positive or negative regulatory signals provided by the stromal cells. Schmitt and Zúñiga-Pflücker attempted to address this question by comparing S17 and OP9 directly in their culture system. Although unmodified OP9 and S17 stromal cells appeared to function equally well in generating large numbers of B cells, OP9-DL1 stromal cells were vastly superior in their ability to generate DP T cells compared to S17-DL1. It is not clear at what stage of T cell differentiation the difference between these two stromal cell lines first becomes apparent. The failure to generate significant numbers of DP T cells could result from diversion of the T/NK decision or inefficient maturation of early T cell progenitors. Nevertheless, this result raises the important point that there is more to making T cells than the mere presence or absence of a Notch signal.

Implications for the Role of Notch Signaling during T Cell Development

The above data raise important questions relating to the role of Notch signals during different stages of T cell development. For example, how does the spatial ex-

pression of Notch receptors and ligands within the thymus relate to the fundamental importance of an intact thymic structure during thymopoiesis? It is possible that overall thymic output, or the relative amounts of different T cell subsets, may be determined by the availability of different Notch ligands within the thymus. Uncommitted stem cells or developing T cells may compete for access to Notch ligands, or differentially induce expression of molecules that regulate Notch signaling.

The spatial organization of Notch ligands may also regulate thymocyte maturation by regulating the duration of Notch signals as thymocytes migrate to specific regions within the thymus. It is not clear if developing T cells had access to Delta-1 throughout the culture period in the system reported by Schmitt and Zúñiga-Pflücker. There is some evidence that Notch signals are modulated during normal thymocyte development (Deftos et al., 2000), and conflicting reports have argued that excessive Notch signals during the DP stage can either inhibit or promote maturation beyond the DP stage (Izon et al., 2002). The culture system described by Schmitt and Zúñiga-Pflücker will allow future studies to address these questions definitively by adding or removing different Notch ligands during specific stages of thymocyte development.

Therapeutic Implications?

Adoptive T cell immunotherapy is the term applied to the goal of rejecting tumors by reinfusing specific $CD8^+$ T cells that have been isolated from the patient and expanded in vitro on antigen plus IL-2 (Greenberg and Riddell, 1999). Can the culture system described by Schmitt and Zúñiga-Pflücker for generating T cells from stem cells be applied to provide either T cells of a desired anti-tumor specificity or diverse T cell populations for immunodeficiency patients? Some T cell immunodeficiencies such as Di George syndrome are due to defects in thymus stromal cell development and patients with normal hematopoietic stem cells benefit from epithelial thymus grafts. Would Delta-1 transfected stromal cells cocultured with peripheral blood stem cells provide a pool of T cells for engraftment? For best immunoprotection, the stromal cell would have to be HLA matched to the patient so that positive selection on the appropriate HLA alleles would select the most useful T cell repertoire to protect the host and so that complete self-tolerance would be induced to guard against self-reactivity and resulting autoimmune disease. We do not know whether CD4 and CD8 positive selection actually occurs in these "simple" cocultures, nor whether complete negative selection could be imposed. It is likely that thymus architecture is specially designed for purging the T cell repertoire of dangerous anti-self-reactive clones, and for promoting the development of regulatory T cells to control immune responses. Until we know more, using an in vitro system to reconstitute the T cell compartment seems a risky procedure.

Could the new system be used in a more targeted way to generate new effector T cells with a desirable anti-tumor specificity? For this one would need (1) knowledge of the tumor target epitope, say for example HLA-A2/melanA, (2) a Delta-1 bearing stromal line that carries the A2 gene and is deficient in class I presentation, for example as a result of TAP deficiency, and (3) altered peptide ligands that are related to the target epitope, bind HLA-A2, and that serve to positively select $CD8^+$ T cells with high affinity for the tumor antigen. Patient stem cells cocultured with such a stromal cell line plus peptide would result in the selective maturation of $CD8^+$ T cells on the A2/peptide complexes (since these are the only ligands present on the stromal cells). $CD8^+$ T cells that mature could be expanded on tumor antigen plus IL-2 for infusion into the patient. This procedure may be less risky than growing a diverse T cell pool, and it gets around the problem that cancer patients may have no or only low affinity T cells, but again, we do not know whether the T cell repertoire is subject to rules of selection in these cultures.

The novel approach of using Notch ligand-expressing stromal cell lines to allow T cell commitment and development in culture immediately provides a wonderful tool for detailed experimental analysis of the differentiation program. Clinical applications will have to wait.

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